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Research Article

Taxonomic and phylogenetic insights into dipteran-parasitizing *Ophiocordyceps*: Descriptions of two new species and a new record from China and Laos

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Abstract

Ophiocordyceps species are renowned for their ecological roles and medicinal potential, yet their diversity on dipteran hosts remains insufficiently documented. Here, we investigated the diversity of dipteran-parasitizing *Ophiocordyceps* from China and Laos, describing two novel taxa—*O. calliphoridarum* and *O. laosensis*—and reporting *O. muscae* as a new record for Laos. Phylogenetic analyses based on a five-locus dataset (ITS, *nr*LSU, *tef-1α*, *rpb1*, and *rpb2*) strongly support the recognition of the two new species within the *O. dipterigena* complex of the hymenostilboid clade. *Ophiocordyceps calliphoridarum* is closely related to *O. muscidarum* but differs by parasitizing *Lucilia caesar* (Calliphoridae) rather than the housefly (Muscidae) and by possessing significantly larger asci and part-spores. *O. laosensis* closely resembles *O. muscae* but can be distinguished by its elongated perithecial ostioles and large asci and part-spores. Additionally, the asexual morph of *O. muscidarum* was newly described. These findings broaden our knowledge of the taxonomy and diversity of dipteran-parasitizing *Ophiocordyceps*, and further corroborate the phylogenetic monophyly of this lineage, thereby offering valuable insights into the co-evolutionary relationships between *Ophiocordyceps* fungi and their dipteran hosts.

Key words: Diptera, monophyly, multi-locus phylogeny, *Ophiocordyceps*, taxonomy

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Introduction

The genus *Ophiocordyceps* was established by Petch in 1931 based on specimens parasitizing cockroaches with *O. blattae* as the type species. It is the most species-rich genus within the family Ophiocordycipitaceae, comprising more than 400 described species (http://www.indexfungorum.org) with a

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broad global distribution across tropical, subtropical, and temperate regions (Sung et al. 2007; Wijayawardene et al. 2017; Luangsa-ard et al. 2018; Araújo and Hughes 2019; Khonsanit et al. 2019; Wei et al. 2020; Dai et al. 2024; Xu et al. 2025; Guan et al. 2025).

Species of the genus *Ophiocordyceps* possess considerable value in medicine and biological control. The well-known Chinese caterpillar fungus *O. sinensis* (Berk.) G.H. Sung et al. has long been used in traditional medicine for its nutritional and therapeutic properties (Liang 2007; Dong et al. 2016). Other species such as *O. xuefengensis*, *O. liangshanensis* also exhibit notable antibacterial, antitumor, and antiviral activities (Yu et al. 2010; Wu et al. 2019). The *O. unilateralis* complex, known for manipulating the behavior of infected ants—the so-called "zombie-ant fungi"—provides a unique model for studying host specificity and parasitic manipulation (Evans et al. 2011; Will et al. 2020). Additionally, the *Hirsutella* anamorphs of *Ophiocordyceps*, such as *H. minnesotensis* and *H. rhossiliensis*, are effective biocontrol agents against nematodes and other agricultural pests (Rath 2000).

Ophiocordyceps species are frequently characterized by brightly and dull-colored stromata and perithecia that are either completely immersed or superficially distributed. Their ascospores are typically filiform, multi-septate, and often fragment into part-spores (Sung et al. 2007). Members of *Ophiocordyceps* exhibit an exceptionally broad host range, parasitizing insects from various orders, including Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, Neuroptera, Odonata, and Orthoptera. They are capable of infecting hosts at all developmental stages—larval (*Ophiocordyceps sinensis* etc.), pupal (*O. cochlidiicola* etc.) and adult (*O. nutans* etc.) (Sung et al. 2007; Shrestha et al. 2016; Luangsa-ard et al. 2018; Tasanathai et al. 2022; Tang et al. 2023; Dai et al. 2024; Mongkolsamrit et al. 2024; Chang et al. 2026).

Although *Ophiocordyceps* exhibits remarkable species diversity, with a wide range of host associations, species parasitizing adult dipteran insects remain relatively few. Considering the important role of dipteran pest control in agriculture and human activities, investigating the diversity of dipteran-parasitizing *Ophiocordyceps* species is of great significance. Dipteran insects serve crucial ecological roles in nature, yet certain species, such as fruit flies (Tephritidae), act as agricultural pests (Hudiwaku et al. 2021; Scolari et al. 2021), while others, notably mosquitoes, function as major disease vectors (Nainu et al. 2022). Additionally, some dipterans contribute beneficially to ecosystem regulation. Tachinid flies such as *Anagonia lasiophthalma*, *Exorista segregata*, and *Pentatomophaga latifascia* participate in natural pest control (Chen et al. 2020; Hammami et al. 2023; Martins et al. 2023). *Ophiocordyceps dipterigena*, initially reported by Berkeley and Broome (1873), represents the earliest known species parasitizing adult Diptera, characterized by light brown to brown stromata emerging from the host thorax and bearing sexual ascocarps at the stromatal tips. Advances in molecular techniques have since led to the discovery and updated taxonomic understanding of 10 *Ophiocordyceps* species associated with adult flies, which clustered to a well-supported monophyletic clade and referred to as the '*O. dipterigena*' complex within the hymenostilboid clade (Mongkolsamrit et al. 2025; Yang et al. 2025).

This study investigates the diversity of *Ophiocordyceps* fungi associated with dipteran hosts. Through field surveys conducted in China and neighboring Laos,

several dipteran-parasitizing *Ophiocordyceps* species were discovered. Detailed morphological examinations and multi-locus phylogenetic analyses were performed. We propose two new species and a new record of *Ophiocordyceps*, and provide supplementary taxonomic information for the known species *O. muscidarum*. Each taxon is described in detail with key diagnostic features, and the species diversity of dipteran-parasitizing *Ophiocordyceps* is further discussed.

Materials and methods

Specimen collection

Fungal specimens were collected from the forests of China and neighboring Laos. Habitat information, such as altitude and latitude, was recorded at the time of collection. Following in situ macrophotography to document the parasitic morphology, the specimens were promptly placed in dry plastic containers and transported to the laboratory for further examination. All specimens were dried and subsequently deposited in the Mycological Herbarium of Guizhou Medical University (**GMB**), China.

Morphological study

Initially, macro-morphological features of the specimens were documented, with the color, and shape of the stromata, followed by observation under a dissecting microscope (Nikon SMZ745T, Nikon Corporation, Japan) to describe the fertile part and perithecia Sections of the fertile head were mounted on glass slides with a drop of lactic acid and lactophenol cotton blue, covered with a cover slip, and observed and photographed under a Leica DM2500 compound microscope (Leica Microsystems, Germany) for detailed measurements of perithecia, asci, peridium, apical cap, ascospores, and secondary ascospores. Asexual structures, comprising conidiogenous cells, phialides, and conidia, were observed on the surfaces of the host fly's body and legs. The synnemata, typically arising from the host abdomen, were occasionally covered with *Hymenostilbe*-like phialides. The abdomen and legs were examined under a stereomicroscope to assess the presence of these phialides, and their morphological characteristics were recorded.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted directly from the wild specimen. Each specimen was thoroughly homogenized using a sterile rod, and total genomic DNA was then extracted using a commercial genomic DNA purification kit (Qiagen GmbH, Hilden, Germany). The quality-checked DNA was stored at −20 °C for subsequent analyses.

Polymerase chain reaction (PCR) was carried out in a 25 μL reaction volume, consisting of 1 μl DNA template, 1 μl each of forward and reverse primers (10 μM each), 9.5 μl ddH_2O, and 12.5 μL of 2× Taq PCR Master Mix (TIANGEN, China). The following nuclear loci were amplified and sequenced: the internal transcribed spacer (ITS) region of ribosomal DNA, the translation elongation factor 1-α gene (*tef-1α*), and the genes encoding the largest and second largest

subunits of RNA polymerase II (*rpb1* and *rpb2*). The primer pairs used were as follows: ITS5/ITS4 for ITS (White et al. 1990), LROR/LR5 for *nr*LSU (Vilgalys and Hester 1990), 983F/2218R for *tef-1α* (Rehner and Buckley 2005), CRPB1/RPB1Cr_oph for *rpb1* (Castlebury et al. 2004; Araújo et al. 2018), and fRPB2-5F2/fRPB2-7cR for *rpb2* (Liu et al. 1999; Sung et al. 2007). The amplification protocol consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 1 min, and extension at 72 °C for 55 s, with a final extension at 72 °C for 10 min. PCR reactions were performed using a Bio-Rad T100 thermal cycler (Bio-Rad, USA). Amplified products were examined by electrophoresis on a 1% agarose gel stained with ethidium bromide in TBE buffer. The PCR products were subsequently purified and sequenced on the ABI3730XL automatic sequence analyzer (Sangong biotech, China).

Host identification

Host identification was performed using a combination of morphological examination and molecular sequencing. For molecular identification, the mitochondrial *cox1* gene was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994). The PCR reaction system and thermal cycling program were the same as those used for the ITS sequences of the same samples. PCR products were sequenced using an ABI 3730XL automatic sequencer. The resulting sequences were analyzed using BLAST method to determine the host species.

Phylogenetic analyses

Phylogenetic analyses were conducted using a combined dataset of five loci: ITS, *nr*LSU, *tef-1α*, *rpb1* and *rpb2*. Sequences newly generated in this study were combined with reference sequences obtained from previous publications and retrieved from GenBank (Table 1). Sequence alignment was performed with MEGA v6.06 (Tamura et al. 2013). The alignment parameters for ITS and *nr*LSU were set to default. For *tef-1α*, *rpb1*, and *rpb2*, only the exon regions were used. After alignment, sequences were examined in codon mode to ensure proper translation into proteins and to avoid the presence of stop codons or other errors caused by sequencing mistakes, with manual correction performed when necessary. *Paraisaria gracilis* and *P. phuwiangensis* were selected as the outgroup.

The final concatenated alignment had a total length of 4795 bp, with the following distribution across loci: ITS (863 bp), *nr*LSU (993 bp), *tef-1α* (1001 bp), *rpb1* (741 bp), and *rpb2* (1197 bp). This 5-gene supermix was further partitioned into 11 distinct segments: one segment each for ITS and *nr*LSU, along with nine additional segments corresponding to the three codon positions within the protein-coding genes *tef-1α*, *rpb1*, and *rpb2*. The optimal partitioning scheme and evolutionary models for the 11 predefined partitions were determined using PartitionFinder2 (Lanfear et al. 2017), employing a greedy algorithm and the Akaike information criterion. The analysis yielded the following 10 partitions: Partition 1—ITS:, Partition 2—*nr*LSU, Partitions 3–5—*tef-1α* codon1, codon 2 and codon 3, Partition 6—*rpb1* codon1, *rpb2* codon1; Partitions 7—*rpb1* codon2, *rpb2* codon2; Partitions 8—*rpb1* codon3, and Partition 9— *rpb2* codon3.

Table 1. List of taxa included in the phylogenetic analysis and their GenBank accession numbers.

Species	Host/Substrate	Strain	Genbank accession number					Reference
			ITS	*nr*LSU	*tef-1α*	*rpb1*	*rpb2*	
Ophiocordyceps blattae	Blattodea	BCC 38241	–	MT512657	MT533485	MT533479	–	Mongkolsamrit et al. 2021
O. communis	Blattodea, Termitidae	BCC 1842	MH754726	MH753680	MK284266	MK214110	MK214096	Tasanathai et al. 2019
O. communis	Blattodea, Termitidae	BCC 1874	MH754725	MH753679	MK284267	MK214109	MK214095	Tasanathai et al. 2019
O. termiticola	Blattodea, Termitidae	BCC 1920^{T}	MH754724	MH753678	MK284265	MK214108	MK214094	Tasanathai et al. 2019
O. termiticola	Blattodea, Termitidae	BCC 1770	–	MH753677	MK284264	MK214107	MK214093	Tasanathai et al. 2019
O. aphodii	Coleoptera	ARSEF 5498	–	DQ518755	DQ522323	–	DQ522419	Spatafora et al. 2007
O. appendiculata	Coleoptera	NBRC 106959	JN943325	JN941412	AB968578	JN992463	AB968540	Ban et al. 2015
O. brunneipunctata	Coleoptera	OSC 128576	–	DQ518756	DQ522324	DQ522369	DQ522420	Spatafora et al. 2007
O. curculionum	Coleoptera	OSC 151910	–	KJ878885	–	KJ878999	–	Quandt et al. 2014
O. houaynhangensis	Coleoptera	MY11460	MH092892	MH092908	MH092899	–	–	Crous et al. 2018
O. houaynhangensis	Coleoptera	MY11461	MH092893	MH092909	MH092900	–	–	Crous et al. 2018
O. annulata	Coleoptera	CEM 303	–	–	KJ878962	KJ878995	–	Quandt et al. 2014
Paraisaria phuwiangensis	Coleoptera	TBRC 9709^{T}	MK192015	MK192057	MK214082	MK214086	–	Mongkolsamrit et al. 2019
O. calliphoridarum	**Diptera (*Lucilia caesar*)**	**GMB 3129^{T}**	**PX219623**	**PX225004**	**PX225635**	**–**	**PX245454**	**This study**
O. calliphoridarum	**Diptera (*Lucilia caesar*)**	**GMB 3130**	**PX219624**	**PX225005**	**PX225636**	**–**	**PX245455**	**This study**
O. calliphoridarum	**Diptera (*Lucilia caesar*)**	**GMB 3131**	**PX219625**	**PX225006**	**PX225637**	**–**	**PX245456**	**This study**
O. dipterigena	Diptera	OSC_151911	–	KJ878886	KJ878966	KJ879000	–	Quandt et al. 2014
O. dipterigena	Diptera	OSC 151912	–	KJ878887	KJ878967	KJ879001	–	Quandt et al. 2014
O. dipterigena	Diptera	HUA 186102	–	KJ917568	–	KF658664	KC610715	Sanjuan et al. 2015
O. dipterigena	Diptera	Hymdip995	–	KJ917573	–	–	KC610712	Sanjuan et al. 2015
O. floriformis	Diptera (*Clephydroneura* sp.)	BBH 27634	PV170894	OP493200	OP503163	–	OP503164	Mongkolsamrit et al. 2025
O. floriformis	Diptera (*Clephydroneura* sp.)	BBH 51295	PV170895	PV257643	PV274276	–	PV274287	Mongkolsamrit et al. 2025
O. forquignonii	Diptera	OSC 151908	–	KJ878889	–	KJ879003	KJ878947	Quandt et al. 2014
O. forquignonii	Diptera	OSC 151902	–	KJ878876	–	KJ878991	KJ878945	Quandt et al. 2014
O. globiceps	Diptera	MFLUCC 18-0495	MH725815	MH725829	MH727387	–	–	Xiao et al. 2019
O. globiceps	Diptera	MFLU 18-0661^{T}	MH725816	MH725830	MH727388		–	Xiao et al. 2019
O. hemisphaerica	Diptera	FLOR 59525^{T}	KX197233	–	–	–	–	Hyde et al. 2016
O. hemisphaerica	Diptera	FLOR 59542	KX197234	–	–	–	–	Hyde et al. 2016
O. laosensis	**Diptera (*Musca* sp.)**	**GMB 3137^{T}**	**PX219630**	**PX225011**	**PX225642**	**PX225644**	**PX245461**	**This study**
O. laosensis	**Diptera (*Musca* sp.)**	**GMB 3138**	**PX219631**	**PX225012**	**PX225643**	**PX225645**	**PX245462**	**This study**
O. muscae	Diptera (*Musca domestica*)	BCC 73607	PV170896	PV257645	PV274277	–	PV274289	Mongkolsamrit et al. 2025
O. muscae	Diptera (*Musca domestica*)	BCC 73616^{T}	PV170897	PV257646	PV274278	–	PV274290	Mongkolsamrit et al. 2025
***O. musca*e**	**Diptera (*Musca* sp.)**	**GMB 3135**	**PX219628**	**PX225009**	**PX225640**	**–**	**PX245459**	**This study**
O. muscae	**Diptera (*Musca* sp.)**	**GMB 3136**	**PX219629**	**PX225010**	**PX225641**	**–**	**PX245460**	**This study**
O. muscidarum	Diptera (Muscidae)	HKAS 132178^{T}	PQ423676	PQ423695	PQ675604	–	PQ569900	Yang et al. 2025
O. muscidarum	Diptera (Muscidae)	HKAS 132275	PQ423677	PQ423696	PQ675605	–	PQ569901	Yang et al. 2025
O. muscidarum	**Diptera (*Coenosia* sp.)**	**GMB 3132**	**PX219626**	**PX225007**	**PX225638**	**–**	**PX245457**	**This study**
O. muscidarum	**Diptera (Coenosia sp.)**	**GMB 3133**	**PX219627**	**PX225008**	**PX225639**	**–**	**PX245458**	**This study**
O. philippinensis	Diptera	LOD PF 4565^{T}	OQ641807	OQ641808	OQ660303	–	–	Crous et al. 2023

Species	Host/Substrate	Strain	Genbank accession number					Reference
			ITS	*nr*LSU	*tef-1α*	*rpb1*	*rpb2*	
O. philippinensis	Diptera	BCC 79225	PV170899	PV257648	PV274280	—	—	Mongkolsamrit et al. 2025
O. philippinensis	Diptera	BCC 78339	PV170900	PV257649	PV274281	—	—	Mongkolsamrit et al. 2025
O. philippinensis	Diptera	BCC 22048	PV170898	PV257647	PV274279	—	PV274291	Mongkolsamrit et al. 2025
O. philippinensis	Diptera	BCC 79871	—	—	PV274282	—	PV274292	Mongkolsamrit et al. 2025
O. philippinensis	Diptera	BCC 79872	—	PV257650	PV274283	—	PV274293	Mongkolsamrit et al. 2025
O. tabani	Diptera	BCC 45127	PV170901	PV257652	—	—	PV339938	Mongkolsamrit et al. 2025
O. tabani	Diptera	BCC 39918	—	PV257651	PV274284	—	—	Mongkolsamrit et al. 2025
O. thilosuensis	Diptera	BCC 46607	PV170903	PV257654	PV274286	—	—	Mongkolsamrit et al. 2025
O. thilosuensis	Diptera	BCC 47494	PV170902	PV257653	PV274285	—	PV274294	Mongkolsamrit et al. 2025
O. anshunensis	Hemiptera	GMBC 3026^{T}	PP583071	PP577938	PP681121	PP681111	PP681116	Guan et al. 2025
O. anshunensis	Hemiptera	GMBC 3027	PP583072	PP577939	PP681122	PP681112	PP681117	Guan et al. 2025
O. aphrophoridarum	Hemiptera	MFLU 20-0641^{T}	MW139322	MW139330	MW160163	MW160167	MW160165	Yang et al. 2021
O. asiana	Hemiptera	GMBC 3023	PP583068	PP577935	PP681118	PP681108	PP681113	Guan et al. 2025
O. asiana	Hemiptera	NBRC 101749	AB968408	JN941429	AB968589	JN992446	AB968550	Ban et al. 2015
O. fulgoromorphila	Hemiptera	HUA 186139^{T}	—	KC610760	KC610729	KF658676	KC610719	Sanjuan et al. 2015
O. fulgoromorphila	Hemiptera	HUA 186142	—	KC610761	KC610730	KF658677	—	Sanjuan et al. 2015
O. longissima	Hemiptera	NBRC 106965	AB968406	AB968420	AB968584	—	AB968546	Ban et al. 2015
O. neonutans	Hemiptera	KEL113^{T}	KX197239	—	—	—	—	Friedrich et al. 2018
O. neonutans	Hemiptera	KEL114	KX197241	—	—	—	—	Friedrich et al. 2018
O. neonutans	Hemiptera	KEL142	KX197244	—	—	—	—	Friedrich et al. 2018
O. nutans	Hemiptera	GMBC 3024	PP583069	PP577936	PP681119	PP681109	PP681114	Guan et al. 2025
O. sobolifera	Hemiptera	NBRC 106967	AB968409	AB968422	AB968590	—	AB968551	Ban et al. 2015
O. sobolifera	Hemiptera	TNS F18521	—	KJ878898	KJ878979	KJ879013	—	Quandt et al. 2014
O. tessaratomidarum	Hemiptera	MY10830^{T}	—	MW280218	MW292434	—	—	Khao-ngam et al. 2021
O. tessaratomidarum	Hemiptera	GMBC 3025	PP583070	PP577937	PP681120	PP681110	PP681115	Guan et al. 2025
O. tricentri	Hemiptera	NBRC 106968	AB968410	AB968423	AB968593	—	AB968554	Ban et al. 2015
O. yakusimensis	Hemiptera	HMAS 199604	—	KJ878902	—	KJ879018	KJ878953	Quandt et al. 2014
O. nutans	Hemiptera	T70	AB366623	—	—	—	—	Sasaki et al. 2008
O. asiana	Hemiptera	BCC 84234^{T}	MW285708	MW280201	MW292438	—	—	Khao-ngam et al. 2021
O. australis	Hymenoptera	HUA 186104	—	KC610763	KC610733	—	KC610713	Sanjuan et al. 2015
O. australis	Hymenoptera	Ophaus926	KF937350	KC610765	KC610735	KF658662	—	Sanjuan et al. 2015
O. buquetii	Hymenoptera	HMAS 199617	—	KJ878905	KJ878985	KJ879020	—	Quandt et al. 2014
O. cylindrospora	Hymenoptera	MFLU 17-1961	MG553635	MG553652	—	——	MG647029	Hyde et al. 2018
O. evansii	Hymenoptera	HUA 186159^{T}	KP200889	KC610770	KC610736	MK863830	—	Sanjuan et al. 2015
O. evansii	Hymenoptera	HUA 186163	KP200890	KC610771	KC610737	MK863831	—	Sanjuan et al. 2015
O. formicarum	Hymenoptera	TNS F18565	—	KJ878888	KJ878968	KJ879002	KJ878946	Quandt et al. 2014
O. granospora	Hymenoptera	BCC 82255^{T}	MH028143	MH028156	MH028183	MH028168	MH028177	Khonsanit et al. 2019
O. granospora	Hymenoptera	BCC 82256	MH028144	MH028157	—	MH028169	MH028178	Khonsanit et al. 2019
O. irangiensis	Hymenoptera	NBRC 101400	JN943335	JN941426	—	JN992449	—	Schoch et al. 2012
O. irangiensis	Hymenoptera	BCC 82795	MH028142	—	MH028186	MH028164	MH028174	Khonsanit et al. 2019
O. khaoyaiensis	Hymenoptera	BCC 82796^{T}	MH028150	MH028153	MH028187	MH028165	MH028175	Khonsanit et al. 2019
O. khaoyaiensis	Hymenoptera	BCC 82797	MH028151	MH028154	MH028188	—	MH028176	Khonsanit et al. 2019
O. lloydii	Hymenoptera	OSC 151913	—	KJ878891	KJ878970	KJ879004	KJ878948	Quandt et al. 2014

Species	Host/Substrate	Strain	Genbank accession number					Reference
			ITS	*nr*LSU	*tef-1α*	*rpb1*	*rpb2*	
O. megacuculla	Hymenoptera	BCC 82262	MH028146	MH028161	MH028191	MH028172	MH028180	Khonsanit et al. 2019
O. megacuculla	Hymenoptera	BCC 82984^{T}	MH028148	MH028162	MH028192		MH028181	Khonsanit et al. 2019
O. myrmecophila	Hymenoptera	MFLU 16-2912	MF351726	MF372585	MF372759	–	–	Xiao et al. 2017
O. pseudolloydii	Hymenoptera	MFLU 22-0266	OQ127360	OQ127394	OQ186385	OQ186434	OQ186408	Wei et al. 2022
O. sphecocephala	Hymenoptera	NHJ4224	GU723778	–	GU797131	–	–	Luangsa-ard et al. 2011
O. thanathonensis	Hymenoptera	HKAS 102442	OQ127361	OQ127395	OQ186386	–	OQ186409	Wei et al. 2022
O. thanathonensis	Hymenoptera	MFLU 16-2910	MF850375	MF850377	MF872614	MF872616	–	Xiao et al. 2017
O. vespulae	Hymenoptera	GACP2017079^{T}	–	MN044859	MN117076		MN107548	Long et al. 2021
O. liangshanensis	Lepidoptera	YFCC 8578	MT774249	MT774226	MT774247	MT774233	MT774240	Wang et al. 2021
O. macroacicularis	Lepidoptera	NBRC 100685^{T}	AB968400	AB968416	AB968574	–	AB968536	Ban et al. 2015
O. sinensis	Lepidoptera	EFCC 7287	JN049854	EF468827	EF468767	EF468874	EF468924	Sung et al. 2007
O. unituberculata	Lepidoptera	YFCC HU1301^{T}	KY923212	KY923212	KY923216	KY923218	KY923220	Wang et al. 2018
Paraisaria gracilis	Lepidoptera	GMBC 3066	PQ787761	PQ785779	PQ789222	PQ789225	PQ789228	Chen et al. 2025
O. odonatae	Odonata (Dragonfly)	TNS F18563	AB104725	KJ878877	–	–	–	Quandt et al. 2014

Phylogenetic analyses of the combined alignment were performed with RAx-ML-HPC BlackBox v8.2.12 (Stamatakis 2014) via the CIPRES Science Gateway with 1000 bootstrap replicates. Additional maximum likelihood (ML) analysis was carried out using IQ-TREE v2.1.3 (Minh et al. 2020) with ultrafast bootstrapping to estimate branch support. Bayesian inference (BI) method was performed using MrBayes v3.2.7a (Ronquist et al. 2012) for five million generations. The nucleotide substitution models for each partition in the three analytical methods mentioned above were automatically determined and output by PartitionFinder 2. After the analyses were completed, the bootstrap support values and posterior probabilities obtained from the three different methods were simultaneously annotated on the phylogenetic tree.

Results

Phylogenetic analyses

Fifty-four *Ophiocordyceps* taxa were included in the phylogenetic analyses conducted in this study. The dataset consisted of 99 samples in total, nine of which were newly generated. Phylogenetic trees reconstructed under both ML and BI criteria showed congruent topologies and supported the recognition of the previous well-defined clade —Hymenostilboid clade (Mongkolsamrit et al. 2025; Yang et al. 2025) (Fig. 1). This clade was mainly composed of five subclades, namely *O. dipterigena* complex (parasitizing flies, *Diptera*), *O. myrmecophila* complex (parasitizing ants, *Hymenoptera*), *O. irangiensis* complex (parasitizing wasps and spittlebugs, *Hymenoptera* and *Hemiptera*), *O. australis* complex (parasitizing ants and wasps, *Hymenoptera*), and *O. nutans* complex (parasitizing stink bugs, *Hemiptera*). This phylogenetic structure is highly consistent with previous reports (Mongkolsamrit et al. 2025; Yang et al. 2025).

The two new *Ophiocordyceps* species described in this study, together with 9 previously reported dipteran-parasitizing *Ophiocordyceps* species, were all clustered within the monophyletic group—'*O. dipterigena*' complex, which was defined by Mongkolsamrit et al. (2025) to represent *Ophiocordyceps* species

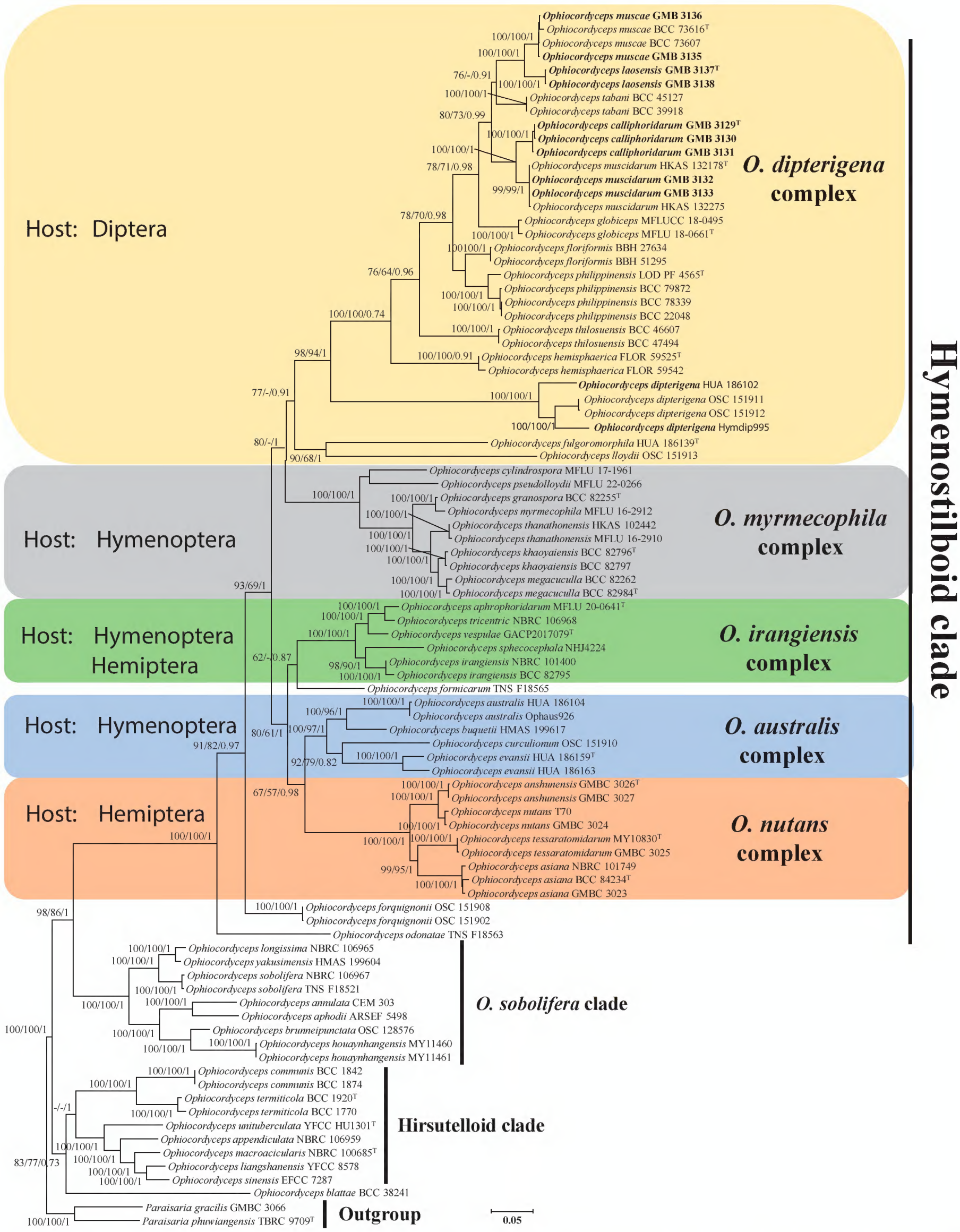

Figure 1. Phylogenetic relationships of *Ophiocordyceps* based on combined partial ITS + *nr*LSU + *tef-1α* + *rpb1* + *rpb2* sequences. Numbers at the branches indicate support values (IQ-TREE-BS/RAxML-BS/BI-PP) above 50%/50%/0.5. Ex-type materials are marked with "T".

parasitizing dipteran hosts. Two new species, *Ophiocordyceps calliphoridarum* and *O. laosensis*, were each resolved as distinct, well-supported lineages. *Ophiocordyceps calliphoridarum* formed a sister clade to *O. muscidarum*, whereas

O. laosensis was recovered as sister to *O. muscae* (Fig. 1). The newly recorded *O. muscae* from Laos clustered within the same clade as conspecific samples, showing no detectable genetic divergence.

Taxonomy

***Ophiocordyceps calliphoridarum* Y. Wang & Y.D. Dai, sp. nov.**

MycoBank No: 860783

Fig. 2

Etymology. The epithet "calliphoridarum" refers to its host belonging to the family Calliphoridae (Diptera).

Holotype. CHINA, • Jilin Province, Dunhua City (43.41°N, 128.33°E, alt. 685 m), on *Lucilia caesar* (the species was identified by *cox1* sequence), on the trunk, 26th Aug. 2024, collected by Kun Zhang, Yao Wang and Yongdong Dai (GMB 3129).

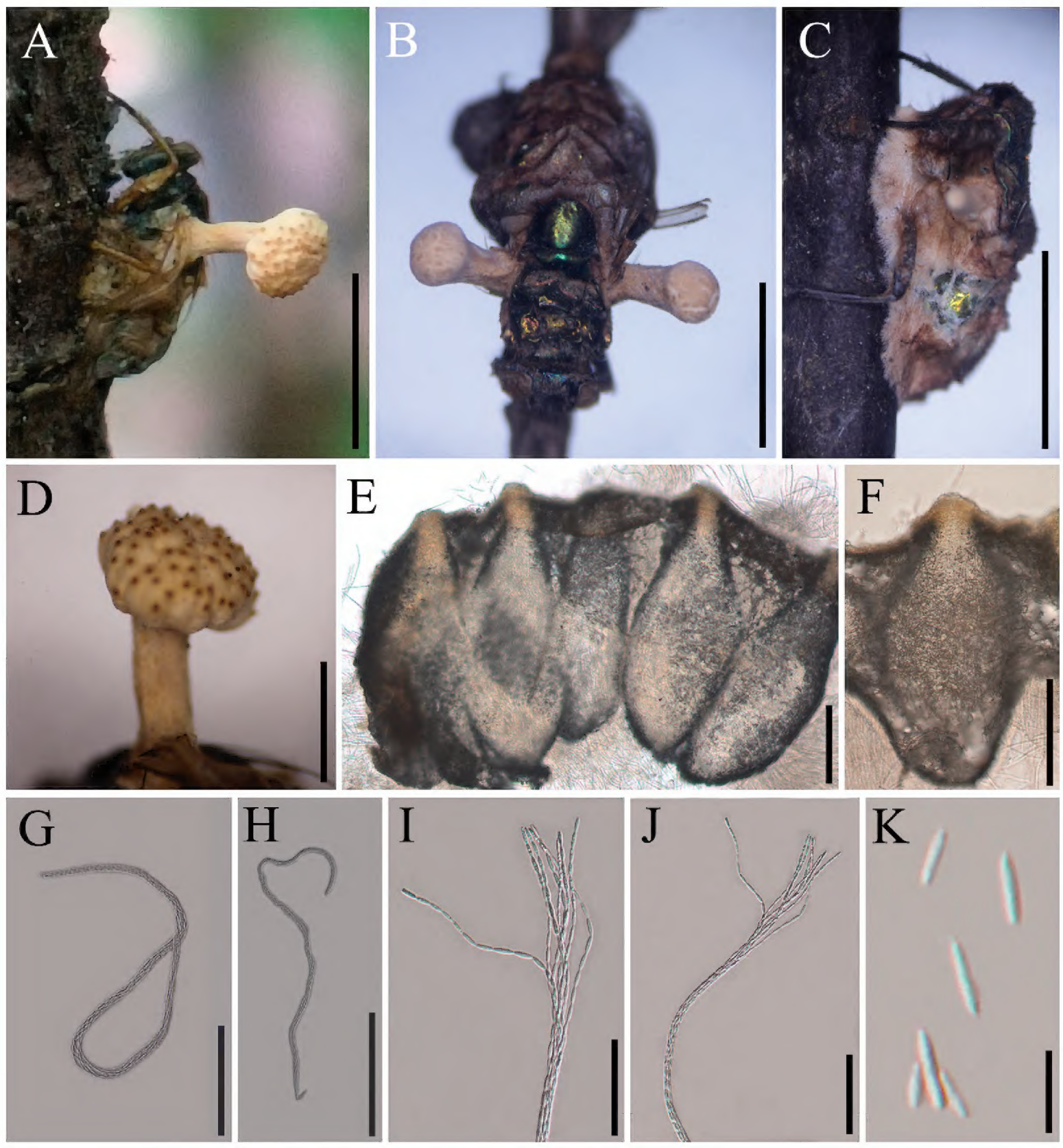

Figure 2. *Ophiocordyceps calliphoridarum*. **A, B.** Fungus on fly (*Lucilia caesar*, Calliphoridae, Diptera); **C.** The early-stage infected host by the *Ophiocordyceps* fungus; **D.** Stromata with spherical fertile part; **E, F.** Perithecia; **G, H.** Asci; **I–K.** Part-spores. Scale bars: 5 mm (**A–C**); 2 mm (**D**); 200 μm (**E, F**); 100 μm (**G, H**); 50 μm (**I, J**); 10 μm (**K**).

Description. Sexual morph: ***Stromata*** stipitate, one or several arising from the prothorax and back region of the host, capitate, unbranched, pale yellow, 3–6 mm long, 0.5–1.5 mm wide with a fertile apex (Fig. 2A, B). ***Fertile heads*** hemispherical to globoid, upper surface slightly convex, moderate orange yellow, located at the terminal part of stipes, 0.5–1.5 mm thick, 1.5–3 mm diam (Fig. 2D). ***Perithecia*** 620–750 × 180–300 µm ($\bar{x}$ = 706 × 235 µm, n = 20), immersed, flask-shaped. ***Asci*** 230–510 × 6.1–8.3 µm ($\bar{x}$ = 386 × 7.4 µm, n = 20), 8-spored, hyaline, cylindrical. ***Apical cap*** 2.6–4.5 × 3.8–5.6 µm ($\bar{x}$ = 3.6 × 4.3 µm, n = 20), thick, hyaline. ***Ascospores*** filiform, multi-septate, breaking into part-spores, cylindrical to fusoid, 8.5–11.5 × 1.5–3.5 µm ($\bar{x}$ = 9.8 × 2.6 µm, n = 60).
Asexual morph: Not observed in natural substrates.

Host. *Lucilia caesar* (Calliphoridae, Diptera).

Habitat. The specimens were found on the trunk of a dicotyledonous plant.

Other material examined. CHINA. • Jilin Province, Dunhua City (43.36°N, 128.27°E, alt. 640 m), on *Lucilia caesar*, 28th Aug 2024, collected by Kun Zhang, Yao Wang and Yongdong Dai (GMB 3130, GMB 3131).

Notes. From a phylogenetic perspective, *Ophiocordyceps calliphoridarum* is closely related to *O. muscidarum*, yet it is distinguished by the formation of an independent clade with high statistical support (Fig. 1; 99/99/1.0). Both species parasitize dipteran hosts; however, *O. calliphoridarum* infects *Lucilia caesar* (Calliphoridae), whereas *O. muscidarum* targets the housefly (Muscidae). Micromorphological examinations further reveal that the asci and part-spores of *O. calliphoridarum* are significantly larger than those of *O. muscidarum* (Table 2). Based on integrated morphological and phylogenetic evidence, *Ophiocordyceps calliphoridarum* is proposed herein as a new taxonomic taxon.

Ophiocordyceps laosensis **Y. Wang & Y.H. Guan, sp. nov.**

MycoBank No: 860784

Fig. 3

Etymology. The epithet refers to the country (Laos) where the type specimen was collected.

Holotype. LAOS, • Oudomxay Province, Muang Xay City (20.26°N, 101.38°E, alt. 1032 m), parasitic on an adult of the housefly (*Musca* sp.), collected on leaves, 6 Aug 2024, Yao Wang (GMB 3137).

Table 2. Summary of morphological comparison among *Ophiocordyceps calliphoridarum*, *O. laosensis* and related taxa.

Species	Host	Stromata (mm)	Distribution	Perithecia (µm)	Asci (µm)	Part-spores (µm)	References
O. calliphoridarum	**Diptera (Calliphoridae)**	**Single or Multiple, 3–6 × 0.5–1.5**	**China**	**flask-shaped, 620–750 × 180–300**	**Cylindrical, 230–510 × 6.1–8.3**	**Cylindrical, 8.5–11.5 × 1.5–3.5**	**This study**
O. laosensis	**Diptera (Muscidae)**	**Multiple, 5–9 × 0.5–1.3**	**Laos**	**flask-shaped, 320–1300 × 150–380**	**Cylindrical, 480–570 × 4.0–12.0**	**Cylindrical, 11–15 × 2.0–4.7**	**This study**
O. muscae	Diptera (Muscidae)	Multiple, 4–8 × 0.5–1.5	Thailand	Ovoid to obclavate, 820 –1100 × 320–400	Cylindrical, up to 720 long, 4–5 wide	Cylindrical to fusoid, 10–13 × 1.5–2	Mongkolsamrit et al. 2025
O. muscae	**Diptera** (Muscidae)	**Multiple, 5–11 × 0.3–1.5**	**Laos**	**Ovoid to obclavate, 380 –540 × 150–220**	**Cylindrical, 430–610 × 5.8–7.0**	**—**	**This study**
O. muscidarum	Diptera (Muscidae)	Single or Multiple, 5–7 × 1–4	China	flask-shaped, 570–760 × 190–310	Cylindrical,280–430 × 5.4–7.5	Fusiform, 7–10.5 × 1.6–2.5	Yang et al. 2025
O. muscidarum	**Diptera (Muscidae)**	**Multiple, 2–3 × 0.4–1.7**	**China**	**flask-shaped, 600–780 × 130–240**	**Cylindrical, 310–400 × 4.2–6.8**	**Cylindrical, 7.2–9.5 × 1.0–2.6**	**This study**

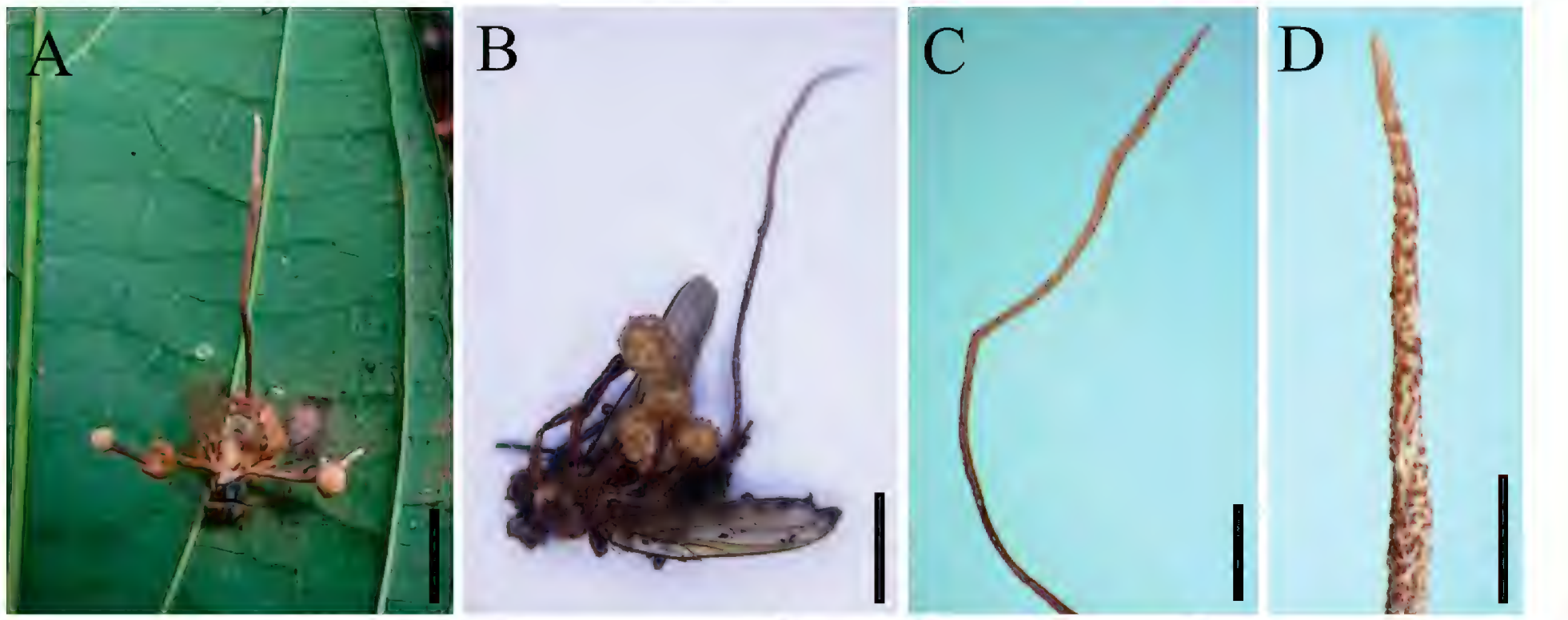

Figure 3. *Ophiocordyceps laosensis*. **A, B.** Fungus on housefly (*Musca* sp., Muscidae, Diptera); **C, D.** Columnar stromata bearing wart-like protuberances; **E, F.** Perithecia; **G, H.** Asci; **I, J.** Part-spores. Scale bars: 5 mm (**A, B**); 1 mm (**C, D**); 500 μm (**E**); 100 μm (**F–H**); 50 μm (**I**); 20 μm (**J**).

Description. Sexual morph: ***Stromata*** stipitate, one or several arising from the thorax region of the host, beneath the wings, capitate, unbranched, brown, 5–9 mm long, 0.5–1.3 mm wide with a fertile apex (Fig. 3A, B). ***Fertile heads*** globoid, surface convex, Orange-yellow to brown, located at the terminal part of stipes, 0.8–1.7 mm thick, 1.6–3.8 mm diam (Fig. 3B). ***Perithecia*** 320–1300 × 150–380 μm ($\overline{x}$ = 864 × 283 μm, n = 20), immersed, flask-shaped. ***Asci*** 480–570 × 4.0–12.0 μm ($\overline{x}$ = 516 × 9.2 μm, n = 20), 8-spored, hyaline, cylindrical. ***Apical cap*** 4.2–8.5 × 5.8–11.7 μm ($\overline{x}$ = 6.4 × 9.3 μm, n = 20), thick, hyaline. ***Ascospores*** filiform, multi-septate, breaking into many (~8) part-spores, cylindrical, 11–15 × 2.0–4.7 μm ($\overline{x}$ = 13.2 × 3.5 μm, n = 20). **Asexual morph:** Not observed in natural substrates.

Host. *Musca* sp. (Muscidae, Diptera).

Habitat. The specimens were found on the underside of a dicotyledonous leaf from a forest plant.

Other material examined. LAOS, • Oudomxay Province, Muang Xay City (20.12°N, 101.06°E, alt. 986 m), parasitic on an adult of *Musca* sp. (Muscidae, Diptera), collected on leaves, 7 Aug 2024, Yao Wang (GMB 3138).

Notes. *Ophiocordyceps laosensis* possesses two distinct types of stromata: globose and columnar. The globose stromata contain developing asci and as-

cospores, whereas the columnar stromata bear wart-like projections on the surface, which may superficially resemble asci but are, in fact, non-fertile structures.

Phylogenetic analyses revealed that *O. laosensis* formed a distinct lineage within the *O. muscae* core group, with strong statistical support (Fig. 1; 100%/100%/1). *Ophiocordyceps laosensis* closely resembles *O. muscae*, as both species parasitize dipteran hosts. Both species produce rod-shaped stromata capped by fertile heads with nearly spherical surface projections. However, *O. laosensis* can be distinguished from *O. muscae* by its more elongated perithecial ostioles, as well as consistently larger asci and part-spores (Table 2).

Ophiocordyceps muscae **Mongkolsamrit, Liangsiri, Thanakitpipattana & Luangsa-ard, MycoKeys 119: 244 (2025)**

MycoBank No: 858733

Fig. 4

Note. The description and illustrations were based on specimens of *O. muscae* collected in Laos.

Description. Sexual morph: Stromata 5–11 mm long, 0.3–1.5 mm wide, multiple, stipitate, cylindrical, capitate, pale yellow to brown, arising from the thorax region of the host (Fig. 4A, B). ***Fertile heads*** 1.0–1.5 × 1.4–2.6 mm, dark brown, with asexual morph at the apex (Fig. 4B, C). ***Perithecia*** 380–540 × 150–220 µm ($\bar{x}$ = 430 × 176 µm, n = 20), immersed, ovoid to obclavate. ***Asci*** 430–610 × 5.8–7.0 µm ($\bar{x}$ = 554 × 6.3 µm, n = 20), 8-spored, hyaline, cylindrical. ***Apical cap***

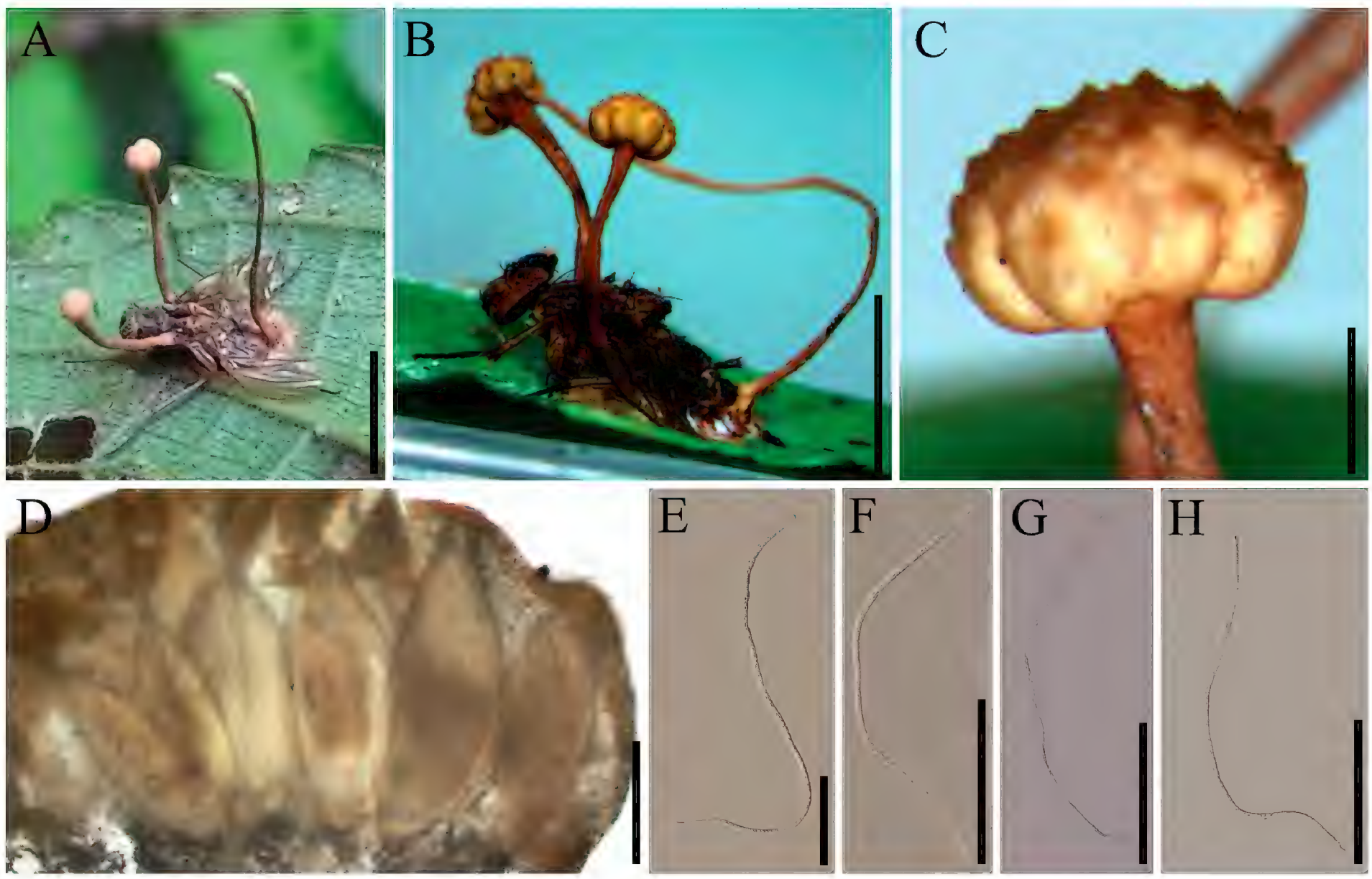

Figure 4. *Ophiocordyceps muscae*. **A, B.** Fungus on housefly (*Musca domestica*, Muscidae, Diptera); **C.** Stromata; **D.** Perithecia; **E–H.** Asci. Scale bars: 5 mm (**A, B**); 1 mm (**C**); 200 µm (**D–H**).

3.2–5.7 × 5.5–10.3 µm ($\bar{x}$ = 4.6 × 8.2 µm, n = 20), thick, hyaline. **Asexual morph:** Not observed in natural substrates.

Materials examined. LAOS, • Oudomxay Province, Namkat Yolapa Resort (19.93°N, 100.36°E, alt. 1069 m), parasitic on *Musca* sp. (Muscidae, Diptera) on the leaves, 6 Aug 2025, Yao Wang (GMB 3135, GMB 3136).

Notes. *Ophiocordyceps muscae* was first reported by Mongkolsamrit et al. (2025) based on specimens collected in Thailand. Phylogenetic analysis confirmed that the newly collected Laotian specimens (GMB 3135, GMB 3136) cluster within the *O. muscae* clade with high statistical support (Fig. 1; 100%/100%/1). Morphometric comparisons revealed that the perithecia of these specimens are smaller than those of the type material; however, no significant differences were observed in macroscopic morphology or other reproductive structures. Based on integrated molecular and morphological evidence, the newly collected specimens are identified as *O. muscae*. This collection represents the first documented occurrence of *O. muscae* in Laos.

***Ophiocordyceps muscidarum* Y. P. Xiao, K.D. Hyde & Y. Yang, MycoKeys 117: 298 (2025)**

MycoBank No: 902879
Index Fungorum: IF902879
Facesoffungi Number: FoF16766
Fig. 5

Note. The description and illustrations are based on specimens of *O. muscidarum* collected during this study.

Description. Sexual morph: Stromata 2–3 mm long, 0.4–1.7 mm wide, multiple, stipitate, cylindrical, capitate, pale yellow, arising from both sides of the host's body (Fig. 5A–D). ***Fertile heads*** 1.4–2.2 × 2.6–4.8 mm, discoid, pale yellow, with asexual morph at the apex (Fig. 5C). ***Perithecia*** 600–780 × 130–240 µm ($\bar{x}$ = 683 × 194 µm, n = 20), immersed, bowling-pin-shaped, ovoid to obclavate. ***Asci*** 310–400 × 4.2–6.8 µm ($\bar{x}$ = 346 ×5.3 µm, n = 50), 8-spored, hyaline, cylindrical. ***Apical cap*** 4.8–8.2 × 2.8–4.3 µm ($\bar{x}$ = 6.5 × 3.4µm, n = 50), thick, hyaline. **Asexual morph:** Asexual spore-producing structures were observed on the host's hind limbs. ***Conidiogenous cells*** *Hymenostilbe*-like, phialidic, forming a hymenial layer. ***Phialides*** single, 8.6–16.5 × 3.5–7.4 µm ($\bar{x}$ = 13.6 × 5.7µm, n = 20). Middle portion strongly swollen, usually tapering abruptly to a slender neck 0.3–1.0 µm diam. ***Conidia*** 3.6–6.8 × 1.5– 3.1 µm ($\bar{x}$ = 5.1 × 2.6 µm, n = 20), 1-cell, hyaline, fusiform.

Materials examined. CHINA. • Heilongjiang Province, Yichun City (48.13°N, 129.61°E, alt. 832 m), on *Coenosia* sp., 23 July 2025, collected by Kun Zhang (GMB 3132, GMB 3133). CHINA. • Jilin Province, Dunhua City (44.12°N, 128.63°E, alt. 596 m), on *Helina* sp., Aug 2025, collected by Kun Zhang (GMB 3134). CHINA. • Jilin Province, Dunhua City (43.62°N, 127.54°E, alt. 610 m), on an adult muscid fly (Diptera: Muscidae), Aug 2025, collected by Kun Zhang (GMB 3141).

Notes. *Ophiocordyceps muscidarum* was first described by Yang et al. (2025). Phylogenetic analyses showed that our newly collected specimens (GMB 3132, GMB 3133) clustered within the *O. muscidarum* clade with strong support (Fig. 1; 99/99/1), exhibiting no genetic differentiation. Morphological compar-

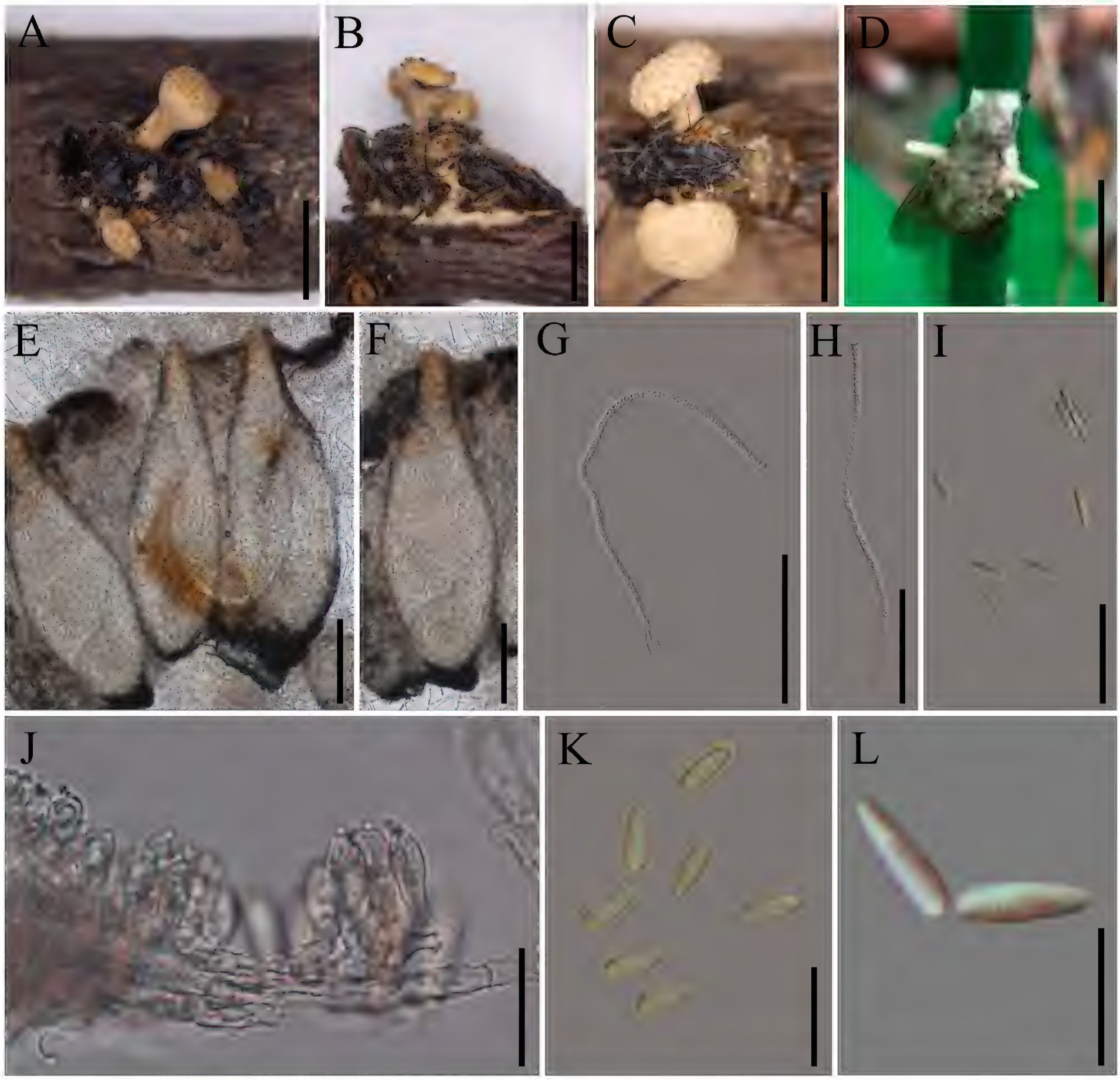

Figure 5. *Ophiocordyceps muscidarum*. **A, B.** Fungus on fly (Muscidae, Diptera); **C.** Stromata; **D.** The early-stage infected host by the *Ophiocordyceps* fungus; **E, F.** Perithecia; **G, H.** Asci; **I.** Part-spores; **J.** Phialides; **K, L.** Conidia. Scale bars: 2 mm (**A, B**); 5 mm (**C, D**); 200 μm (**E, F**); 100 μm (**G, H**); 20 μm (**I, J**); 10 μm (**K**); 5 μm (**L**).

isons revealed no notable differences, except that the stromata observed in our specimens were slightly shorter. Based on the combined molecular and morphological evidence, the specimens were identified as *O. muscidarum*. Furthermore, as the original description lacked information on the asexual stage of this species, partial asexual morphological data for *O. muscidarum* was provided here based on our specimen observations.

Discussion

A systematic taxonomic study was conducted on dipteran-parasitizing *Ophiocordyceps* species based on morphological comparison and a concatenated dataset containing five loci (ITS, *nr*LSU, *tef-1α*, *rpb1*, and *rpb2*) phylogenetic analyses.

One of the new species, *O. calliphoridarum*, is closely related to *O. muscidarum* but differs by parasitizing *Lucilia caesar* (Calliphoridae) rather than the housefly (Muscidae) and by possessing significantly larger asci and part-spores. While *O. laosensis* closely resembles *O. muscae*, it can be distinguished by its elongated perithecial ostioles, as well as its large asci and part-spores. These two newly described species, together with the ten previously reported dipteran-parasitizing *Ophiocordyceps* species, formed a well-supported monophyletic clade—the *O. dipterigena* complex (Mongkolsamrit et al. 2025), providing compelling evidence for the monophyly of *Ophiocordyceps* species infecting dipteran hosts. However, *O. forquignonii* represents an exception, as it parasitizes Diptera but falls outside this clade, being positioned at the terminal of the hymenostilboid clade. Our findings contribute to a better understanding and expansion of the species diversity of this complex. It should be noted that *Ophiocordyceps lacrimoidis* was previously considered part of the *O. dipterigena* complex (Hyde et al. 2016; Mongkolsamrit et al. 2025). However, its phylogenetic position is currently inferred solely from the ITS sequence (Hyde et al. 2016), and based on our analyses, it does not cluster within the *O. dipterigena* complex clade (Suppl. material 1). Although it shares morphological and ecological similarities with members of this complex—being parasitic on Muscidae, possessing a capitated sexual structure with a discoid fertile head, and exhibiting a *Hymenostilbe*-like asexual morph (Hyde et al. 2016), its phylogenetic placement still requires reassessment using additional molecular markers.

Although we have documented morphological differences among the species within *O. dipterigena* complex, these taxa show pronounced considerable morphological convergence at the macroscopic level, typically characterized by pale yellow to brown, clavate stromata bearing fertile ascomata at their apex. Consequently, four species within this clade—*O. dipterigena*, *O. discoideicapitata* (Kobayasi and Shimizu 1982), *Cordyceps muscicola* (Möller 1901; Freire 2015) and *Cordyceps sakishimensis* (Kobayasi and Shimizu 1983), had been reported prior to the application of molecular-assisted identification. This study underscores the critical role of multi-gene data in the species identification, delimitation and systematic phylogeny of cryptic species within this species complex. By conducting five-locus phylogenetic analyses (ITS, *nr*LSU, *tef-1α*, *rpb1*, and *rpb2*), we identified two new species within the *O. dipterigena* complex, as well as a new record from Laos, thereby extending the known geographic distribution, and providing additional biogeographical evidence for the diversity of this group in southeast Asia. The results not only refine the taxonomic framework of the *O. dipterigena* complex but also lay an important foundation for further investigations into the coevolutionary dynamics and ecological adaptations of *Ophiocordyceps* species and their dipteran hosts.

Notably, all specimens of *O. laosensis* and *O. muscae* from Laos were found on the underside of leaves, whereas most *O. calliphoridarum* and *O. muscidarum* specimens from temperate regions of China occurred on stems. Consistent with prior records, tropical members of the *O. dipterigena* complex, including *O. muscae*, *O. floriformis*, and *O. thilosuensis* from Thailand, predominantly inhabit abaxial leaf surfaces (Mongkolsamrit et al. 2025). This distinct microhabitat differentiation is likely influenced by climatic factors: the high temperatures and heavy rainfall of tropical regions may favor development on sheltered leaf undersides, which offer higher humidity and protection from sunlight and precipitation. We suggest that this pattern is not the result of targeted host behav-

ior manipulation, but rather represents an adaptive response to the combined effects of environmental conditions, such as temperature, humidity, and rainfall. Nonetheless, broader sampling and further studies are required to clarify the ecological mechanisms underlying these habitat preferences.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

The authors did not use Artificial Intelligence (AI) or AI-assisted tools during the preparation of my manuscript.

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Author contributions

Yong-dong Dai: Specimen collection, phylogenetic analysis, manuscript writing, and overall review and editing. Yu-Hu Guan: Morphological observation, DNA extraction, PCR amplification, and sequence submission. Sheng-mei Wu: Morphological examination, sequence alignment, and species comparison. Shabana Bibi: Manuscript editing and language polishing. Hui Chen: Image processing and manuscript preparation. Chanhom Loinheuang: Specimen collection and morphological observation. Jiandong Liang: Manuscript revision. Yao Wang: manuscript writing, Image processing, manuscript revision, and quality control.

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Data availability

All of the data that support the findings of this study are available in the main text. All sequences were submitted to GenBank and obtained the accession number.

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Supplementary material 1

Phylogenetic tree of *Ophiocordyceps* with the ITS sequences

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Data type: docx

Explanation note: *Ophiocordyceps lacrimoidis* was previously placed within *O. dipterigena* complex (Mongkolsamrit et al. 2025). However, this species was represented only by the ITS sequence, in our phylogenetic reconstruction, it did not cluster within *O. dipterigena* complex.

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